

## ISOLATION AND PROPERTIES OF GOAT $\beta_2$ -MICROGLOBULIN

**Abstract**—1. Goat  $\beta_2$ -microglobulin was isolated and purified from colostrum.

2. Comparisons of the amino acid composition and amino-terminal sequence of the goat protein with the bovine and human homologues, indicates a high degree of similarity. Both goat and bovine  $\beta_2$ -microglobulins differ slightly in composition from the human molecule, most notably in threonine and proline values.

3. For the first 32 residues, bovine and goat differ only at two positions, one of which is a valyl/isoleucyl substitution consistent with the amino acid compositions. The equivalent goat/human sequence comparison shows seven differences.

4. Immunological studies, using the ELISA method, also confirm the close relatedness of goat and bovine  $\beta_2$ -microglobulin and their more distant relatedness to the human homologue.

### INTRODUCTION

$\beta_2$ -Microglobulin ( $\beta_2$ -m), found free in body fluids, is also the light chain (12,000 mol. wt) of the class I major histocompatibility antigens. The heavy chain (44,000 mol. wt) which spans the plasma membrane, is found on the surface of all nucleated cells in noncovalent association with  $\beta_2$ -m and is highly polymorphic. Based on amino acid sequences,  $\beta_2$ -m is homologous to the  $\alpha_3$ -domain of the heavy chain for the class I histocompatibility antigens and to the immunoglobulin constant-region domains (Peterson *et al.*, 1972; Cunningham *et al.*, 1973; Orr *et al.*, 1979). The class II histocompatibility antigens contain a noncovalently linked heavy ( $\alpha$ ) and light ( $\beta$ ) chains. Although they are not associated with  $\beta_2$ -m, results of amino acid sequence alignment indicate that besides the relationship just mentioned,  $\beta_2$ -m is also homologous to the class II heavy chain  $\alpha_2$ - and the light chain  $\beta_2$ -domains. However, when a computer-aided alignment was made among these Ig-like domains, the  $\beta$ -pleated sheet regions of the tertiary structure called the antibody fold in immunoglobulins, there were indications that class I, class II and  $\beta_2$ -m sequences were comparable in their similarities to each other, but comparatively less similar to immunoglobulin sequences, indicating the existence of two evolutionary sub-groups (Malissen *et al.*, 1983).

In earlier reports from this laboratory, we described the isolation, physical characterization, and complete amino acid sequence of bovine  $\beta_2$ -m (Groves and Greenberg, 1977, 1982; Kumosinski *et al.*, 1981). Only the bovine species has yielded a crystalline  $\beta_2$ -m. The quaternary structure of many IgG domains has been solved by X-ray crystallo-

graphy (Saul *et al.*, 1978) and studies on bovine  $\beta_2$ -m crystals were initiated by Becker *et al.* (1977). Because the goat and bovine diverged in a relatively short period in the evolutionary time scale, it was believed that  $\beta_2$ -m isolated from the goat might be crystallizable, like that from the bovine. In this paper, the isolation and characterization of goat  $\beta_2$ -m is described, and its relationship to the bovine and human proteins compared by chemical and immunological techniques.

### MATERIALS AND METHODS†

#### Casein isolation

Casein was precipitated from skimmed colostrum of goat after dilution with an equal volume of water by the addition of 1 N HCl to pH 4.2, the isoelectric point of goat casein. The casein was collected by centrifugation, washed with an equal volume of water, recovered by centrifugation and then lyophilized.

#### Ion exchange chromatography

Colostrum casein, 24 g, was dissolved in 200 ml 0.005 M sodium phosphate, pH 8.3, by the addition of 0.1 N NaOH to pH 8.3. After the addition of phenylmethylsulfonyl fluoride, 400 mg, the solution was dialyzed overnight against the phosphate buffer at 3°C. The dialysate was applied to a microgranular DEAE-cellulose column (4 × 50 cm), previously equilibrated with the same buffer. After  $\beta_2$ -m was eluted with a buffer change to 0.025 M sodium phosphate, pH 8.3, the resin was washed with water, methanol, and acetone before preparing it for the next run. Apparently, the isolated goat colostrum casein contains neutral fats and phospholipids which build up on the resin and interfere with the flow rate.

After four batches, 24 g casein each, were chromatographed the fractions containing  $\beta_2$ -m were combined (210 mg), dissolved in 6 ml 0.025 M potassium phosphate, pH 5.5 buffer, adjusted to pH 5.5, dialyzed overnight at 3°C against 0.05 M potassium phosphate, pH 5.5, then chromatographed on a CM-cellulose column (2 × 33 cm), previously equilibrated with the latter buffer.

#### Gel filtration

The fraction rich in  $\beta_2$ -m from CM-cellulose was dissolved in 3 ml 0.025 M sodium acetate, pH 5.5 and applied

\*Agricultural Research Service, U.S. Department of Agriculture.

†Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

to a Sephadex G75 superfine column (2 × 28 cm), equilibrated with the same buffer.

#### Polyacrylamide gel electrophoresis

Disc-gel electrophoresis was, according to Davis (1964), at pH 8.6, but in 4 M urea. Electrophoresis (pH 4.3) in 8 M urea was a modification of the method of Reisfeld *et al.* (1962). Polyacrylamide gel electrophoresis in SDS of reduced samples was by the method of Weber and Osborn (1969).

#### Amino acid analysis

Protein samples were hydrolyzed at 110°C for 24 hr with 5.7 N HCl containing phenol (0.05%) in sealed evacuated tubes. The analyses were performed on a Beckman 119CL amino acid analyzer and results reported as molar ratios.

#### Amino acid sequence

Sequencing was carried out on a Beckman 890 C Sequencer using a 0.25 M Quadrol single cleavage program with polybrene and a simultaneous benzene and ethyl acetate wash. Identification of PTH amino acids was accomplished by HPLC and gas chromatography (Greenberg *et al.*, 1984).

#### Immunodiffusion

Ouchterlony double diffusion was carried out in 1.5% agar in barbiturate-HCl buffer pH 8.2,  $\mu = 0.05$  (Berggård, 1961).

#### Reduction and alkylation

Goat and bovine  $\beta_2$ -m were reduced and *S*-carboxymethylated as described by Groves and Greenberg (1982).

#### ELISA

Enzyme immunoassay was carried out in semimicro polystyrene cells, 1.5 ml, with a 10 mm pathlength following a modification of the procedure of Engvall (1980). Coat solution was 0.5 M  $\text{Na}_2\text{CO}_3$ , pH 9.0. Wash solution was 0.05% Tween 20 in 0.9% NaCl. Incubation buffer (PBS) was 0.15 M NaCl, 0.05 M  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{NaN}_3$ , 0.05% Tween 20 titrated to pH 7.2 with KOH. PBS-BSA buffer contained 50 ml PBS and 50 mg bovine serum albumin. Conjugate was alkaline phosphatase labeled anti-rabbit IgG from goat (Miles). Antigens were dissolved in coat solution and 0.2 ml (1  $\mu\text{g}$   $\beta_2$ -m) was added to each cell and incubated 3 hr in a 37°C water bath. The cells were washed three times with 0.2 ml wash solution then incubated 30 min at 25°C with 0.2 ml PBS-BSA on a rotary shaker set at 125 rev/min. The cells were washed twice with 0.2 ml PBS-BSA and filled with 0.2 ml PBS-BSA, then serially diluted by adding to the first cell 50  $\mu\text{l}$  rabbit antibody to bovine  $\beta_2$ -m previously diluted 1/5 with PBS-BSA, mixing and transferring 50  $\mu\text{l}$  to the next cell, etc. The cells were incubated on the shaker at 25°C for 3 hr, then stored at 3°C overnight. The cells were washed three times with wash solution, then 0.2 ml conjugate (1/500 in PBS) was added and they were placed on the shaker for 3 hr at 25°C. After, the cells were washed three times with

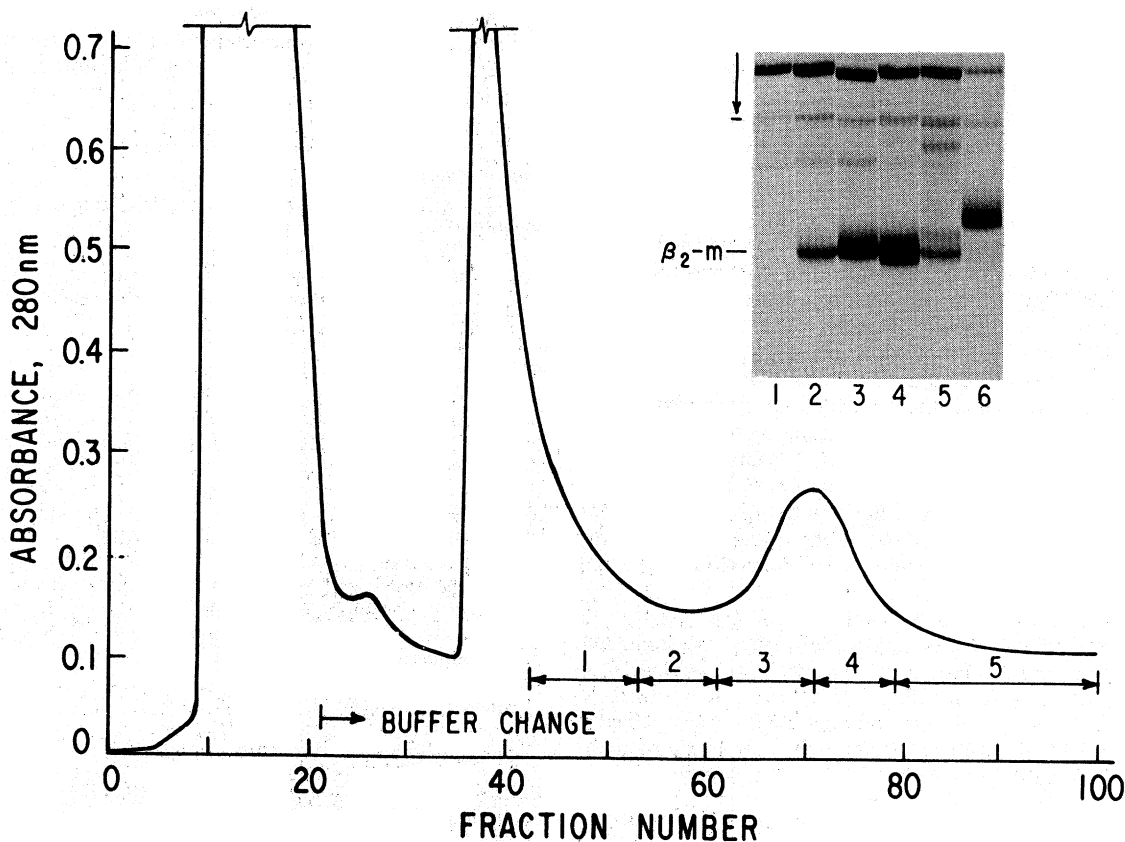


Fig. 1. Colostrum casein chromatographed on a DEAE-cellulose column (4 × 50 cm) starting buffer 0.005 M sodium phosphate, pH 8.3. At fraction 21 buffer changed to 0.025 M. Fractions, 26 ml/tube, were combined as indicated. Fraction 6 represents another small peak, tubes 101–130 (not shown). Disc-gel electrophoretic patterns, pH 4.3, 8 M urea, of the numbered fractions are included.

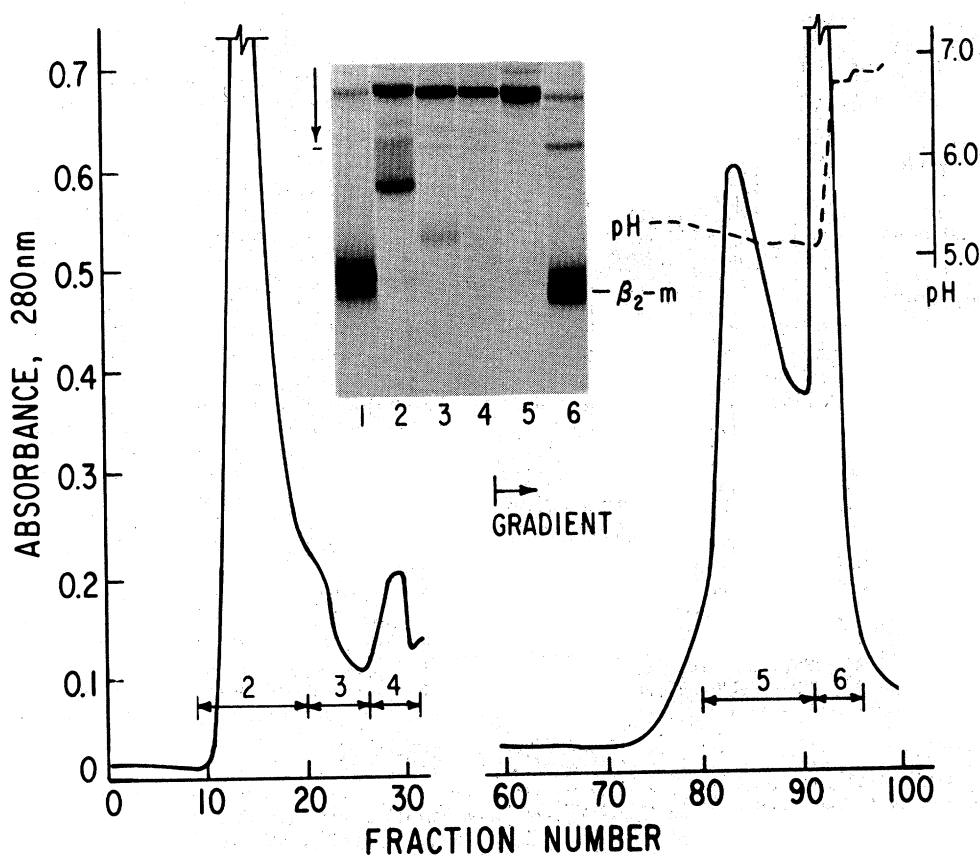


Fig. 2.  $\beta_2$ -m rich fractions (Fig. 1) chromatographed on a CM-cellulose column  $2 \times 33$  cm, starting buffer 0.05 M potassium phosphate, pH 5.5. Fractions, 5 ml/tube, were combined as indicated. At tube 60, a gradient of (1) 200 ml starting buffer; (2) 200 ml (1) with 0.2 M KCl and adjusted to pH 7.6 was begun. The dashed line indicates the pH. A disc-gel electrophoretic pattern, pH 4.3, 8 M urea of the numbered fractions is included, gel 1 is of the protein insoluble in the starting buffer.

wash solution, the alkaline phosphatase color of the conjugate was developed as described by Engvall (1980).

In preliminary experiments, the sodium carbonate coat solution for the bovine  $\beta_2$ -m antigen contained sodium azide and this produced very erratic results.

#### RESULTS AND DISCUSSION

Chromatography of goat colostrum casein on DEAE-cellulose resembles that of bovine colostrum casein (Groves and Greenberg, 1982), except a buffer change to 0.025 M phosphate for goat is required, while bovine  $\beta_2$ -m was eluted with the starting buffer (0.005 M phosphate). Fractions containing goat  $\beta_2$ -m were first identified by Ouchterlony plates, using rabbit anti-bovine  $\beta_2$ -m (not shown). Figure 1 shows a typical chromatogram of goat colostrum casein on DEAE-cellulose and disc gel electrophoretic patterns of combined fractions in which  $\beta_2$ -m is eluted. Fraction 6 pooled from tubes 101-130 (not shown), represents another small peak, and disc gel electrophoresis indicates that its major band corresponds in mobility to goat  $\alpha$ -lactalbumin.

In preliminary experiments it was found that chromatography of goat casein produced variable yields of  $\beta_2$ -m, and in one experiment, after CM-cellulose

fractionation, no  $\beta_2$ -m was obtained. This suggested proteolytic activity due to plasmin or other proteases in goat colostrum; consequently, the protease inhibitor phenylmethylsulfonyl fluoride was added to all goat colostrum casein solutions before chromatography. Although plasmin has been shown to be present in cows' casein (Eigel *et al.*, 1978), it was not a problem in the isolation of bovine  $\beta_2$ -m.

Chromatography on CM-cellulose of fractions from DEAE-cellulose rich in  $\beta_2$ -m (2-5, Fig. 1) is shown in Fig. 2 together with the disc gel electrophoretic patterns of the pooled fractions. Pools 2-5 contain no  $\beta_2$ -m.  $\beta_2$ -Microglobulin is eluted with the pH change indicated by the dashed line. Pool 6 of Fig. 2 was then chromatographed on a gel filtration column as shown in Fig. 3. Pool 4 of Fig. 3, which represents isolated  $\beta_2$ -m, was used for further studies. The yield of goat  $\beta_2$ -m is about half the amount obtained from bovine  $\beta_2$ -m from a given amount of colostrum casein.

Disc gel 1 in Figs. 2 and 3 shows the electrophoretic pattern run with urea for the fractions insoluble in the starting buffers. They appear to contain  $\beta_2$ -m among other proteins. This indicates the presence of an insoluble complex between some of the  $\beta_2$ -m and other proteins in the system.

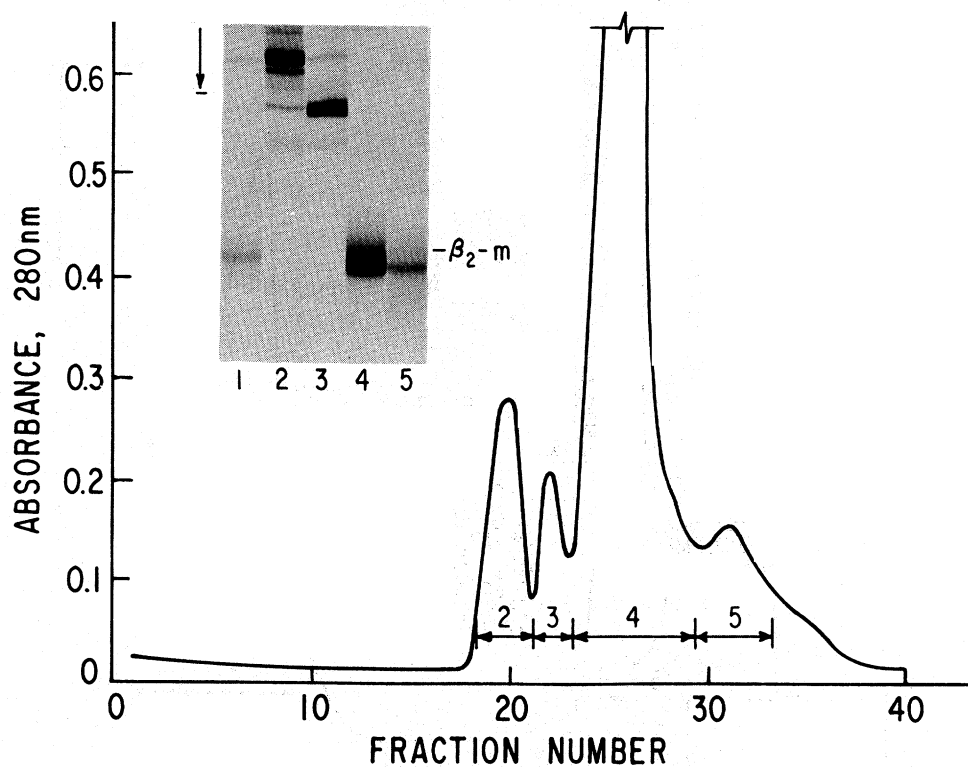


Fig. 3.  $\beta_2$ -m rich fraction 33 mg (Fig. 2, gel 6) on a Sephadex G75, superfine column ( $2 \times 28$  cm) equilibrated with 0.025 M sodium acetate, pH 5.5. Fractions, 5 ml/tube, were combined as indicated and the disc gel electrophoretic pattern, pH 4.3, 8 M urea, of the numbered fractions are shown. Fraction 1 represents protein insoluble in the starting buffer and fraction 4 represents 20 mg pure  $\beta_2$ -m.

Polyacrylamide SDS gel electrophoresis of reduced goat and bovine  $\beta_2$ -microglobulins show single bands with identical mobilities indicating similar molecular weights. Bovine  $\beta_2$ -m has a mol. wt of 11,630, based on its amino acid sequence data (Groves and Greenberg, 1982).

Figure 4 shows the disc gel electrophoretic patterns, pH 4.3, 8 M urea, of goat, bovine, and human  $\beta_2$ -m (gels 1, 3, 5). Bovine and human  $\beta_2$ -microglobulins have a similar net charge, while that of the goat is lower. Reduced and alkylated samples of goat and bovine  $\beta_2$ -m (gels 2 and 4) show relatively slower mobilities than the native proteins. At pH 8.6 in 4 M urea (not shown) bovine  $\beta_2$ -m has the lowest net charge followed by goat and then the human protein.

The amino acid compositions of goat, cow, and human  $\beta_2$ -m are shown in Table 1. Values for bovine and human  $\beta_2$ -m are based on their sequence. Amino acid differences of one are found between goat and bovine for several amino acids, and a difference of two for glycine. A major difference of three and four proline residues is found for goat and bovine vs human  $\beta_2$ -m, respectively. Goat like bovine  $\beta_2$ -m does not contain methionine, which is present in the four other homologues of which the sequences are known (Groves and Greenberg, 1983).

The amino terminal 32 residues of goat  $\beta_2$ -m are compared with the bovine and human homologues in Fig. 5. Only two differences at positions 6 and 7 are

present between the goat and bovine proteins. The valyl/isoleucyl substitution at residue 7 accounts for the amino acid composition difference of those two residues in Table 1. The two di-prolyl sequences at

Table 1. Amino acid composition of  $\beta_2$ -M

	Residues/mole		
	Goat	Bovine <sup>a</sup>	Human <sup>b</sup>
Asp	12	11	12
Thr	3	2	5
Ser	9 <sup>c</sup>	9	9
Glu	12	12	11
Pro	8	9	5
Gly	5	3	3
Ala	2	1	2
Val	6	5	7
Cys	2	2	2
Met	—	—	1
Ile	5	6	5
Leu	8	8	7
Tyr	6	6	6
Phe	4	4	5
Trp	2 <sup>d</sup>	2	2
Lys	8	9	8
His	4	4	4
Arg	4	5	5

<sup>a</sup>Groves and Greenberg (1982).

<sup>b</sup>Cunningham *et al.* (1973).

<sup>c</sup>Uncorrected for destruction.

<sup>d</sup>Assumed value.

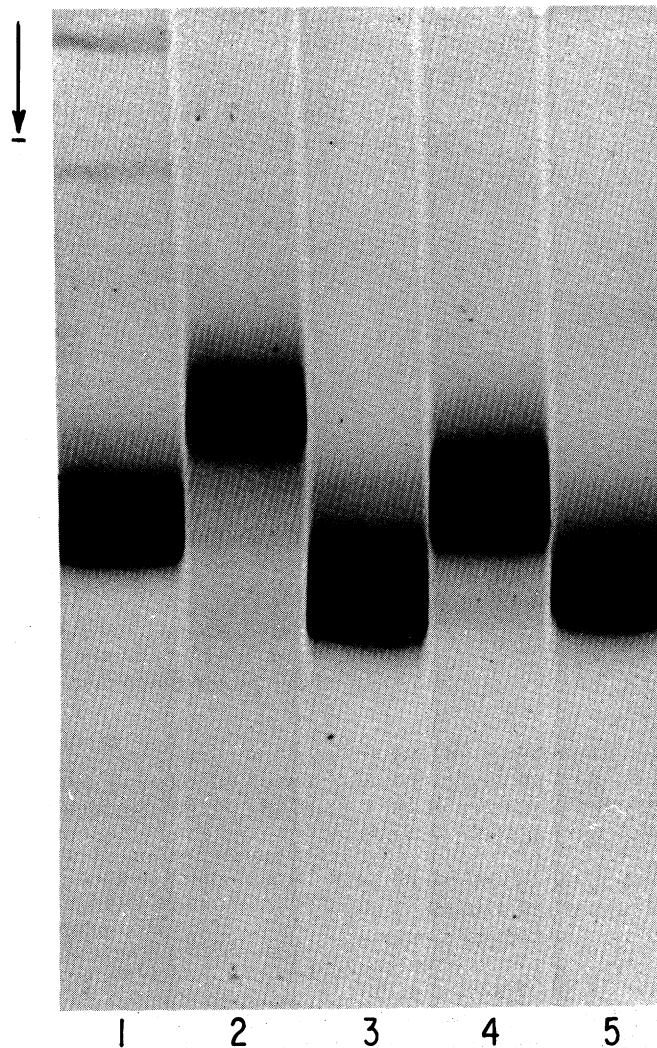


Fig. 4. Disc gel electrophoresis, pH 4.3, 8 M urea, of goat (1), reduced alkylated goat (2), bovine (3), reduced alkylated bovine (4), and human (5)  $\beta_2$ -microglobulins.

	1	5	10	15	20
Goat	Ile-Gln-Arg-Pro-Pro-Glu-Val-Gln-Val-Try-Ser-Arg-His-Pro-Pro-Glu-Asn-Gly-Lys-Pro-				
Bovine	_____ Lys-Ile _____				
Human <sup>a</sup>	_____ Thr _____ Lys-Ile _____ Ala _____ Ser				
	21	25	30		
Goat	Asn-Tyr-Leu-Asn-Cys-Tyr-Val-Tyr-Gly-Phe-His-Pro-				
Bovine	_____				
Human	_____ Phe _____ Ser _____				

<sup>a</sup> Cunningham *et al.* (1973).

Fig. 5.  $\beta_2$ -Microglobulin sequence data.

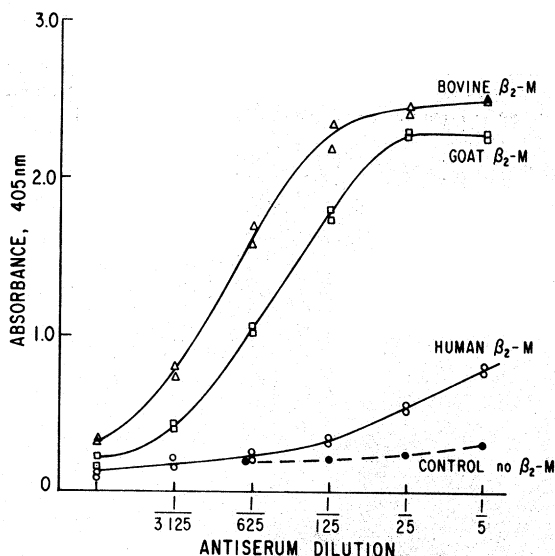


Fig. 6. Titration of rabbit anti-bovine  $\beta_2$ -m in duplicate cells coated with bovine, goat, and human  $\beta_2$ -m. A control with no  $\beta_2$ -m is also shown. Serial dilution of normal rabbit serum gave readings of about 0.1.

residues 4-5 and 14-15, characteristic of bovine  $\beta_2$ -m, are also found in the goat protein. Sequence information was not obtained past residue 32, but it is likely that the third di-prolyl sequence, 32-33, is also present.

It is obvious from Table 1 and Fig. 5, that  $\beta_2$ -m is highly conserved, and as might be expected from their evolutionary history, goat and bovine  $\beta_2$ -microglobulins are more closely related to each other than they are to human  $\beta_2$ -m. Further evidence of this relationship is shown by ELISA assay (Fig. 6). Titration curves using rabbit polyclonal anti-bovine

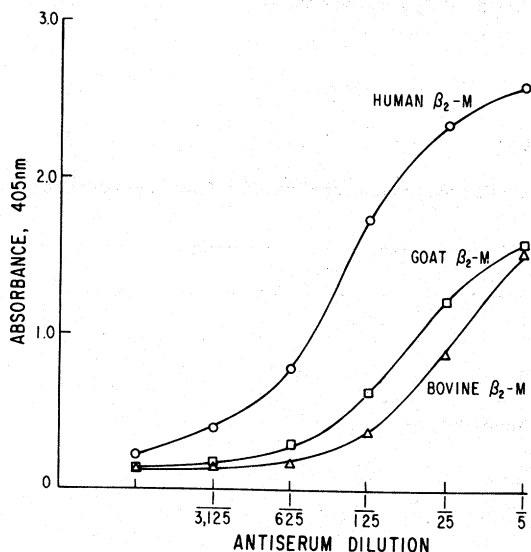


Fig. 7. Titration of rabbit anti-human  $\beta_2$ -m (Calbiochem) in cells coated with human, goat, and bovine  $\beta_2$ -m plotted as averages of duplicates.

$\beta_2$ -m show a strong uptake of antibody when tested against bovine  $\beta_2$ -m, while it is somewhat reduced for goat and only slight for the human protein. When rabbit anti-human  $\beta_2$ -m (Fig. 7), is used instead of the anti-bovine  $\beta_2$ -m, the reverse effect is found: human  $\beta_2$ -m shows a strong uptake while goat closely followed by the bovine protein has significantly less.

In another experiment using anti-bovine  $\beta_2$ -m to compare native with reduced and alkylated bovine  $\beta_2$ -m, the titration curve (not shown) falls about halfway between those for native and goat  $\beta_2$ -microglobulins (Fig. 6). The retention of significant antibody uptake for reduced and alkylated  $\beta_2$ -m compared to the native protein indicates that most of the antibodies are not directed against conformation dependent protein determinants in the  $\beta_2$ -m molecule.

Although goat and bovine  $\beta_2$ -microglobulins are similar in amino acid composition, amino terminal sequence and immunological reactivity, goat  $\beta_2$ -m has resisted our attempts at crystallization, indicating that the small differences observed in these parameters may change the physical properties of the molecule.

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